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Nonselective expression of simian virus 40 large tumor antigen fragments in mouse cells

(recombinant DNA)

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ABSTRACT **To understand the role of various functional domains of simian virus 40 early tumor antigens, we have cloned and introduced into mouse cells portions of early simian virus 40 DNA. Two types of truncated large tumor antigen (33 and 12.3 kilodaltons), as well as small tumor antigen, were identified by immunoprecipitation. Both truncated large tumor antigens have been found to be overproduced with respect to the small tumor antigen, although the 12.3-kilodalton truncated large tumor antigen was more stable than the 33-kilodalton one. Nonviral 53-kilodalton protein was not found associated with either truncated large tumor antigen in immunoprecipitations.**

Simian virus 40 (SV40) encodes two early proteins, small tumor (t) antigen and large tumor (T) antigen (1). The T protein is involved in the onset of viral DNA replication (2), repression of early RNA synthesis (3), transformation of various tissue culture cells (4), activation of host ribosomal genes (5), stimulation of cellular DNA synthesis (6), adenovirus helper function (7), and induction of tumor-specific transplantation antigen (8, 9). Deletion mutants (10) that do not produce t antigen, but only T antigen, are capable of effecting all these functions (11). Although it has been suggested that t antigen may have a helper function or act like a hormone (10), its role is not well understood.

Conventional transformation of cells by SV40 is based on the selection of cells that exhibit altered growth. This requires sufficient viral genetic information or alteration in host-cell gene expression to produce complex phenotypic changes. Some experiments have been carried out to obtain cells that have incorporated intact SV40 DNA without specific selection for the transformed phenotype (12, 13). These experiments were limited by lack of selection methods for cells bearing viral genomes and confined to situations in which intact genes for viral proteins were present. We and others (13, 14) have used selection for covalently linked genes to obtain cell lines that have acquired stably associated SV40 sequences without prior selection for the functions of these sequences.

Recently, separable functional domains within the early proteins have been identified by microinjecting restriction endonuclease-generated DNA fragments of T-antigen-coding segments directly or after cloning plasmid pBR322 into mammalian cells (15). These studies are limited to functions that can be measured at the level of single microinjected cells and for which transient presence of the protein(s) is sufficient. Establishment of cell lines that continuously express truncated forms of T antigen would markedly enhance functional analysis of specific segments of this protein.

We have examined production of fragments of SV40 early protein in mouse cells. For this purpose, we introduced plasmids containing the herpes simplex virus serotype 1 (HSV-1) thymidine kinase (*TK*) gene linked to fragments of SV40 DNA coding for regions of the early proteins into TK-deficient cells and selected for cells stably producing thymidine kinase. A majority of cell clones obtained in this fashion express the unselected SV40 peptides encoded by the SV40 DNA fragment. Various partial peptides were readily detectable, although they had different stabilities. These peptides could be used in locating antigenic domains in the early protein.

MATERIALS AND METHODS

A small plaque isolate of SV40 strain 776 (the variant with a 72-base pair tandem repeat near the origin of replication), bacterial plasmid pBR322, and the *Bam*HI restriction fragment of HSV-1, containing the herpes *TK* gene (16–20), were used as starting materials for DNA in constructions. Restriction endonucleases used were obtained from commercial sources or prepared according to published protocols. DNA restriction enzyme digests and ligations, bacterial transformations, and sequence analyses of plasmids were carried out according to published procedures. The nature of each construction was confirmed by multiple restriction endonuclease digestions and partial sequence analyses. The cells used were mouse LMTK$^-$ cells obtained initially from William Summers and S. Silverstein. Restriction endonucleases and T4 DNA ligase were obtained from commercial sources.

Construction of Bacterial Plasmids. The plasmids used were made following National Institutes of Health containment guidelines. Only a brief description of their constructions is given below.

Plasmid containing the entire early region, pVBETK-1. *Eco*RI linkers (G-G-G-A-A-T-T-C-C-C) were added to *Hpa*II-cut nuclease S1-treated SV40 linear DNA and the DNA was restricted with *Eco*RI/*Bam*HI. The portion of the DNA between 0.72 and 0.14 map units (m.u.), containing the entire early region including the SV40 origin, was cloned between the *Eco*RI and *Bam*HI sites of pBR322. The resulting plasmid, pVBE, was cut with *Bam*HI and the HSV-1 *Bam*HI fragment containing the *TK* gene was inserted to obtain pVBETK-1.

Plasmids containing truncated portions of T antigen and the entire t antigen, pVBtTK-1 and pVBtlTK-1. The construction of pVBtl is as shown in Fig. 1. pVBtl was restricted with *Bam*HI and the HSV-1 *TK Bam*HI fragment was inserted to obtain plasmid pVBtlTK-1. Plasmid pVBETK-1 was restricted with *Hpa* I and the large fragment was self-ligated to obtain pVBtTK-1.

Plasmid containing only a short NH_2-terminal portion of T antigen and the entire t antigen, pVBt2TK-1. The construction

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Abbreviations: SV40, simian virus 40; t and T antigen, small and large tumor antigen, respectively; HSV-1, herpes simplex virus serotype 1; TK, thymidine kinase; m.u., map unit(s); NVT, nonviral T (protein).

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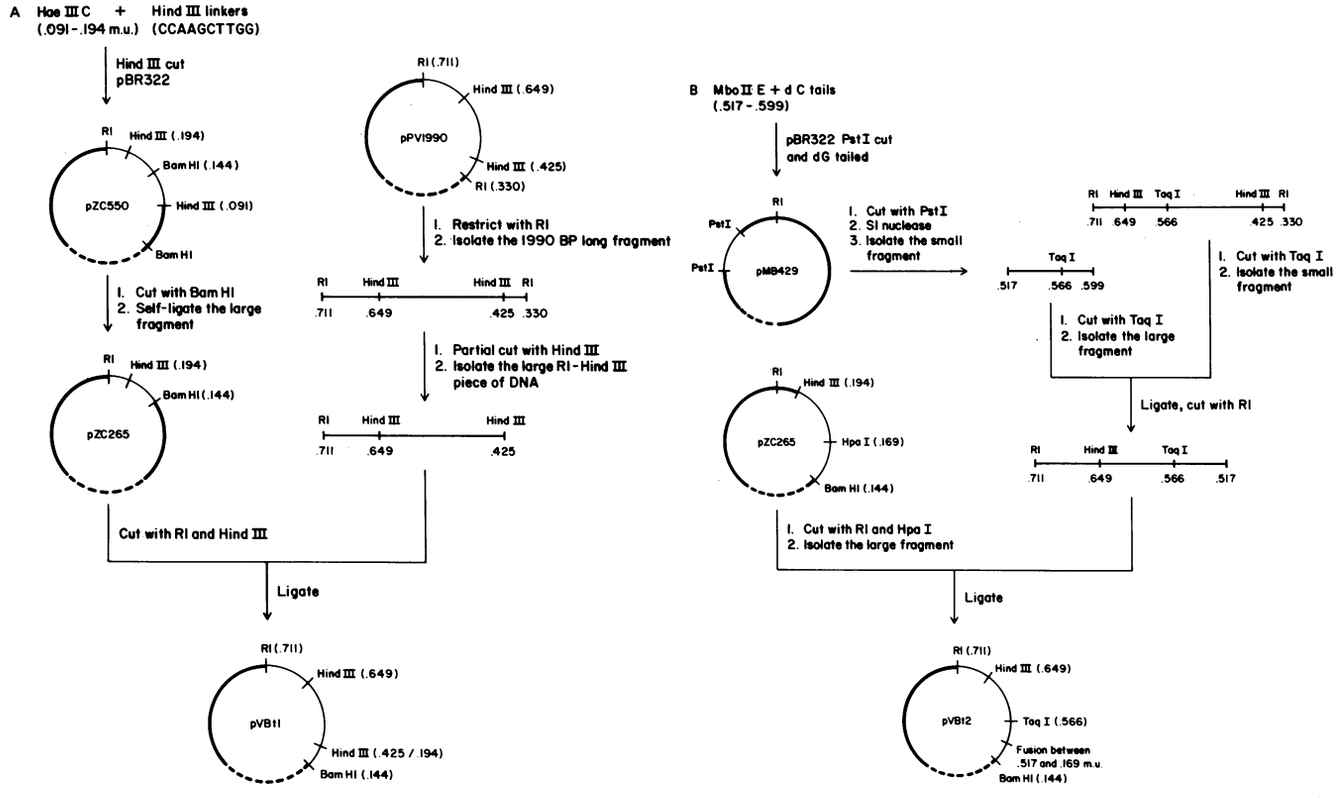

FIG. 1. Construction of plasmids pVBt1 (*A*) and pVBt2 (*B*). Positions of cleavage sites on the SV40 genome are relative to the unique *Eco*RI site. ——, plasmid DNA; ——, SV40 DNA. *Hae* III C is the segment of SV40 DNA between 0.20 and 0.08 m.u.

of pVBt2 is as shown in Fig. 1*B*. This plasmid was cut with *Bam*HI and *TK Bam*HI DNA was inserted to obtain pVBt2TK-1.

Transformation of LMTK$^-$ Cells with Plasmid DNA. Mouse LMTK$^-$ cells were maintained in Dulbecco's modified Eagle's medium/5% fetal calf serum/0.03% glutamine/0.075% $NaHCO_3$ supplemented with penicillin at 100 units/ml and streptomycin at 100 μg/ml. The transformation procedure used was essentially as described (21), except that the carrier DNA used (final concentration, 10 μg/ml) was isolated from LMTK$^-$ cells. The TK$^+$ transformants were selected in Dulbecco's modified Eagle's medium containing hypoxanthine at 15 μg/ml aminopterin at 0.2 μg/ml, and thymidine at 5 μg/ml. Individual clones were picked by using cloning pipettes and grown into mass culture in the same medium.

Preparation of Cell Extracts, Immunoprecipitation, and Gel Electrophoresis. Transformed LMTK$^+$ cells were grown in hypoxanthine/aminopterin/thymidine medium and radiolabeled with [^{35}S]methionine. Cell extracts were prepared and proteins were immunoprecipitated with normal hamster serum or serum from hamsters bearing tumors induced by SV40-transformed cells and analyzed by using polyacrylamide gel electrophoresis as described (22).

RESULTS

During the course of transformation of LMTK$^-$ cells, we observed that TK plasmids (pVBETK, pVBt1TK, pVBt2TK) converted LMTK$^-$ cells into TK$^+$ cells more efficiently than did the HSV-1 TK plasmid alone, containing no SV40 sequences. A 10- to 30-fold increase in the number of transformed colonies was seen when plasmids contained the SV40 origin with or without T-antigen-coding regions. The increased TK transformation was independent of the orientation of the *TK* gene relative to the SV40 origin sequences (23). Similar observations have been made by others using the $CaPO_4$ method of transformation (24) or microinjection (25) of TK$^-$ cells. Recent evidence indicates that this may be a consequence of enhanced transcription (24, 26).

We then examined viral gene expression in cells in which the whole SV40 early gene region and the SV40 early promoter and polyadenylation signal were integrated into pBR322 next to HSV-1 *Bam*HI *TK* gene fragment (plasmid pVBETK-1). A mass culture of transformed cells and nine individual clones were expanded and examined. One clone was totally negative for T antigen and one showed some T-antigen-negative cells while, in the remaining seven clones, every cell was positive for T antigen by immunofluorescence.

Lines of cloned cells were labeled *in vitro* with [S^{35}]methionine and SV40 T and t antigens were immunoprecipitated and examined by electrophoresis on $NaDodSO_4$/polyacrylamide gels. As shown in Fig. 2, these cells synthesized normal-size SV40 T and t antigens, as well as the 55-kilodalton (kDal) cellular nonviral T (NVT) protein (Fig. 2), which has been shown to associate with T antigen (27–30).

Cells transformed by pVBtTK-1, which contains the SV40 early region deleted between 0.37 and 0.17 m.u., produced t antigen but no trace of the expected 40-kDal truncated T antigen (Fig. 3*A*). We have also observed that the NVT protein was not immunoprecipitated by antiserum against viral T antigen. We do not know the reason for the absence of truncated T protein in the cells transformed by pVBtTK-1. It may be that the T-antigen splice is not occurring properly or that the protein itself is unstable.

Cells transformed by pVBt1TK-1, the plasmid containing the first third of the SV40 early region (Fig. 3*B*), did not show T antigen by immunofluorescence and did not demonstrate either normal-sized T antigen or NVT protein but showed t antigen

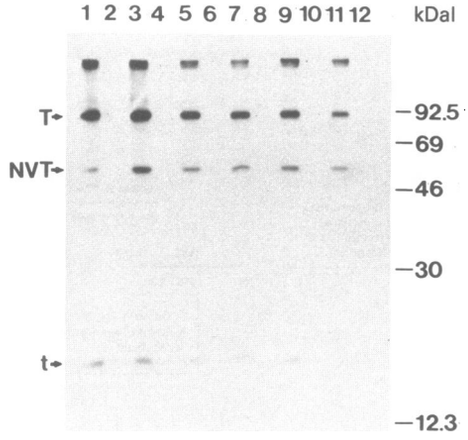

FIG. 2. Synthesis and stability of SV40 T, t, and NVT antigens in pVBETK-1-transformed LMTK$^+$ cells. Cultures were pulse labeled for 30 min with [^{35}S]methionine (50 μCi/ml; 1 Ci = 3.7×10^{10} becquerels) in methionine-free medium (lanes 1 and 2) and radioactive label was chased in the presence of a 100-fold excess of unlabeled methionine for 30 min (lanes 3 and 4), 1 hr (lanes 5 and 6), 2 hr (lanes 7 and 8), 4 hr (lanes 9 and 10), and 20 hr (lanes 11 and 12). Extracts were immunoprecipitated with antitumor serum (lanes 1, 3, 5, 7, 9, and 11) or normal hamster serum (lanes 2, 4, 6, 8, 10, and 12) and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. The band at the top is aggregated material that did not enter the gel.

and a 33-kDal T-antigen-related peptide similar to that produced by a nonviable deletion mutant of SV40 virus (31). Cells transformed by plasmid pVBt2TK-1, which contains only the coding region for t antigen and early splice and polyadenylation sites of SV40, synthesized a normal-sized t antigen and a peptide of ≈13,000 kDal (Fig. 4). Also, the immunoprecipitates from

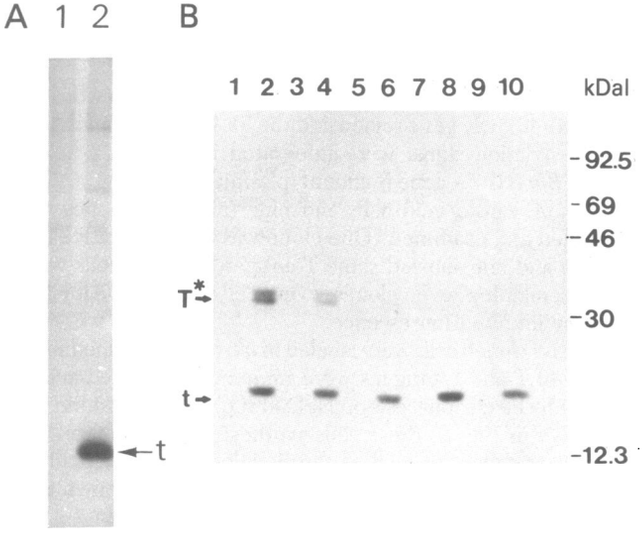

FIG. 3. Synthesis and stability of truncated T and t antigens in pVBt1TK-1- and pVBtTK-1-transformed LMTK$^+$ cells. (*A*) Cultures of pVBtTK-1-transformed cells were labeled in Fig. 2 for 20 min but not chased, and the extracts were immunoprecipitated with normal hamster serum (lane 1) or antitumor serum (lane 2) and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. (*B*) Cultures of pVBt1TK-1-transformed cells were pulse labeled for 1 hr with [^{35}S]methionine (50 μCi/ml; lanes 1 and 2) and chased in the presence of a 100-fold excess of unlabeled methionine for 20 min (lanes 3 and 4), 40 min (lanes 5 and 6), 1 hr (lanes 7 and 8), and 2 hr (lanes 9 and 10). Extracts were immunoprecipitated with antitumor serum (lanes 2, 4, 6, 8, and 10) or normal hamster serum (lanes 1, 3, 5, 7, and 9) and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. Doubling of the truncated T antigen (T*) was an inconsistent finding.

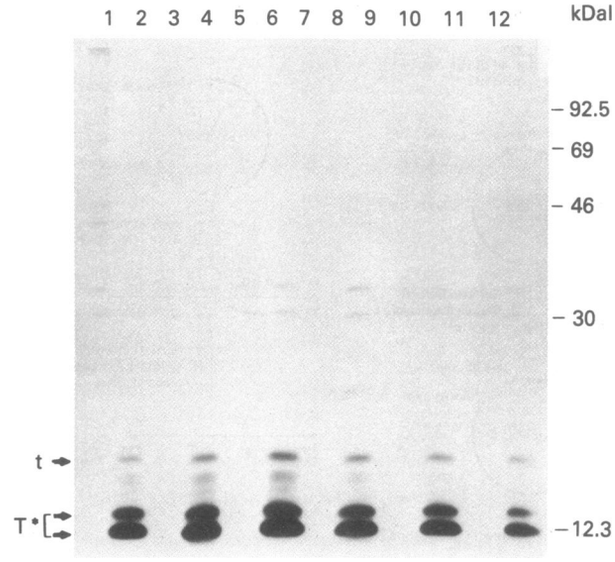

FIG. 4. Stability of t and truncated T antigens in pVBt2TK-1-transformed LMTK$^+$ cells. Cultures were pulse labeled with [^{35}S]methionine (50 μCi/ml; lanes 1 and 2) and chased in the presence of a 100-fold excess of unlabeled methionine for 20 min (lanes 3 and 4), 40 min (lanes 5 and 6), 1 hr (lanes 7 and 8), 2 hr (lanes 9 and 10), and 4 hr (lanes 11 and 12). Extracts were immunoprecipitated with antitumor serum (lanes 2, 4, 6, 8, 10, and 12) or normal hamster serum (lanes 1, 3, 5, 7, 9, and 11) and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. The doublet seen for the 12.3-kDal T protein (T*) may be a gel artifact because a single band is observed in many other immunoprecipitations.

cells transformed by pVBt2TK-1 did not show any NVT protein and the cells showed no T-antigen immunofluorescence.

The identity of the 33-kDal protein of pVBt1TK-1 to authentic truncated T antigen was confirmed by the fact that it was not precipitated by an antitumor serum that is specific for t antigen (22). In addition, the truncated T antigen was not precipitated by antitumor serum specific for the carboxy terminus of T antigen. Antiserum prepared against denatured T antigen (32–34) did react with truncated T antigen and, as shown previously, with t antigen as well (data not shown). Truncated T antigen synthesized by pVBt1TK-1-transformed cells is similar in size to the 33-kDal truncated T antigen synthesized by cells infected by the SV40 deletion mutant dl *1001* (31) and the 33- to 37-kDal truncated T reported here is phosphorylated (data not shown), as was the 33-kDal T antigen synthesized in dl *1001*-infected cells.

To assess the stability of the truncated T antigens, cells were labeled with [^{35}S]methionine and chased with excess unlabeled methionine for various times before immunoprecipitation (Figs. 3 and 4). Methionine radioactivity disappeared relatively rapidly from the 33-kDal truncated T antigen produced by pVBt1TK-1 while the 12-kDal truncated T antigen produced by pVB2TK-1 was relatively stable over the period of the chase, as was intact wild-type T antigen (Figs. 2, 3, and 4). In view of the small number of methionine residues in these truncated T antigens, both the 12- and the 33-kDal truncated T antigens seem to be produced in larger amounts than intact T antigen. This was particularly marked in the case of the 12-kDal truncated T antigen for which a relatively low amount of t antigen was produced. Untransformed cells and cells transformed by the various plasmids produced similar amounts of NVT protein after a brief pulse-labeling period as detected by immunoprecipitation with monoclonal antibody against NVT protein (kindly provided by E. Harlow), in agreement with the evidence

(34) that the accumulation of NVT protein in SV40-transformed cells is due to stabilization rather than accelerated synthesis of the protein.

DISCUSSION

Our results show that it is possible to nonselectively introduce into cells gene fragments coding for truncated or incomplete proteins and to obtain cell lines that stably produce high levels of the proteins. Other workers have noted an apparent epigenetic phenomenon in which cotransfected SV40 viral early regions are not stably expressed in all cells containing the viral gene (3, 5). In the present series of results, the representative clones analyzed from each transformation experiment continued to express the viral proteins for >25–44 passages, as shown by immunoprecipitation and $NaDodSO_4$/polyacrylamide gel electrophoresis. However, only cells transformed with the intact early region of SV40 were examined for immunofluorescence, because the smaller peptides did not give satisfactory immunofluorescence with the available antisera. In those clones expressing a high level of T antigen, every cell showed typical nuclear immunofluorescence. We do not know whether the lack of expression of SV40 T antigen in a minority of clones is a result of DNA rearrangement or an epigenetic phenomenon of the type previously described (14).

The stability of the T-antigen fragments was not simply related to their size and may prove to be a more complex function of the amino acid sequences and potential protein structures formed by various partial peptides. It is curious to observe that plasmid pVBt1TK-1, which produces an unstable 33-kDal protein, has its deletion near the region specifying temperature sensitivity to the T antigen.

The present approach makes it possible to obtain mass cultures of cells overproducing the protein fragments, either for isolation of the fragments or for study of their effects in transformed cells. For example, cells producing the 33-kDal truncated form of T antigen express a SV40-specific transplantation-rejection antigen at the cell surface that can be detected by the lymphocyte mediated cytotoxicity assay (data not shown). Plasmid pVBtTK-1 should be useful in studying the biological role of t antigen. By using transformants generated by the methodology described here, it should be possible to precisely define regions of SV40 T antigens that act as virus-specific transplantation-rejection antigen. In addition, localization of such aspects of T-antigen functions as DNA and NVT protein binding could be investigated.

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